Toxicon 119 (2016) 46–51

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Exploiting the antithrombotic effect of the (pro)thrombin inhibitor bothrojaracin

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ARTICLE INFO

Article history:
Received 22 February 2016
Received in revised form
4 May 2016
Accepted 10 May 2016
Available online 11 May 2016

Keywords:
Bothrops jararaca venom
Bothrojaracin
Thrombin inhibitor
Thrombosis

ABSTRACT

Bothrojaracin is a 27 kDa C-type lectin-like protein from *Bothrops jararaca* snake venom. It behaves as a potent thrombin inhibitor upon high-affinity binding to thrombin exosites. Bothrojaracin also forms a stable complex with prothrombin that can be detected in human plasma. Formation of the zymogen-inhibitor complex severely decreases prothrombin activation and contributes to the anticoagulant activity of bothrojaracin. In the present study, we employed two rodent models to evaluate the antithrombotic effect of bothrojaracin *in vivo*: stasis-induced thrombosis and thrombin-induced pulmonary thromboembolism. It was observed that bothrojaracin interacts with rat prothrombin in plasma. Ex-vivo assays showed stable complex formation even after 24 h of a single bothrojaracin dose. As a result, bothrojaracin showed significant antithrombotic activity in a rat venous thrombosis model elicited by thromboplastin combined with stasis. The antithrombotic activity of bothrojaracin (1 mg/kg) persisted for up to 24 h and it was associated with moderate bleeding as assessed by a tail transection method. Formation of bothrojaracin-prothrombin complex has been also observed following intravenous administration of the inhibitor into mice. As a result, bothrojaracin effectively protected mice from thrombin-induced fatal thromboembolism. We conclude that bothrojaracin is a potent antithrombotic agent *in vivo* and may serve as a prototype for the development of new zymogen-directed drugs that could result in prolonged half-life and possible decreased hemorrhagic risk.

1. Introduction

Cardiovascular diseases are the leading causes of death globally (Pagidipati and Gaziano, 2013). Most of the cardiovascular diseases correlate with increased blood coagulation inside the vessels thus resulting in venous or arterial thrombosis. In addition, increased procoagulant and prothrombotic states are also observed as an underlying condition in several pathological processes including cancer and infectious diseases (Francischetti et al., 2008; Lima and Monteiro, 2013). Thrombin is a serine proteinase that plays a central role in thrombus formation under either physiological or pathological conditions. Therefore, the pivotal role of thrombin in the pathogenesis of cardiovascular diseases makes this enzyme one of the main targets for prevention and treatment of prothrombotic states (Coppens et al., 2012; Kong et al., 2014). Thrombin surface possess two positively charged regions named exosites that play key roles in the specificity and/or recognition of thrombin towards macromolecular substrates (such as fibrinogen, factor V, protease-activated receptor 1) and cofactors (such as factor Va, thrombomodulin) (Bock et al., 2007). Thrombin exosites may also support the recognition of physiological and exogenous inhibitors (Qureshi et al., 2009; Huntington, 2014).

Snake venoms as well as salivary glands from hematophagous animals constitute a major source of molecules capable of modulating hemostasis (Francischetti, 2010; Koh and Kini, 2012). Remarkably, a number of these molecules have been characterized as potent antithrombotic agents *in vivo* in animal models (Calvo et al., 2010; Ma et al., 2013; Waisberg et al., 2014; Chen et al., 2015; Mizurini et al., 2015). Bothrojaracin is a 27-kDa C-type lectin-like protein composed by two distinct chains linked by a single disulfide bridge (Zingali et al., 1993). It is a selective and

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http://dx.doi.org/10.1016/j.toxicon.2016.05.007
0041-0101/

potent thrombin inhibitor that primarily recognizes exosite I thus preventing fibrinogen clotting and platelet aggregation without impairment of the enzyme's catalytic site (Zingali et al., 1993; Monteiro et al., 1999). Interestingly, bothrojaracin recognizes the thrombin zymogen form, prothrombin, through a high-affinity interaction (Arocas et al., 1997). This interaction is mediated by proexosite I, the precursor state of exosite I on prothrombin (Monteiro et al., 2001). Remarkably, complex formation between prothrombin and bothrojaracin results in decreased prothrombin activation by exogenous or physiological activators (Monteiro and Zingali, 2000, 2002). Thus, prothrombin activation by the prothrombinase complex assembled on either synthetic phospholipids or activated platelets is severely impaired upon bothrojaracin-prothrombin complex formation (Monteiro and Zingali, 2002).

Preliminary results from our group suggested that bothrojaracin inhibits thrombosis *in vivo* (Zingali et al., 2005). In the present study, we employed two distinct rodent models in order to characterize the antithrombotic properties of bothrojaracin: stasis-induced thrombosis and thrombin-induced pulmonary thromboembolism. Our data demonstrate that bothrojaracin displays significant and long-lasting antithrombotic effect that was associated with moderate bleeding as compared to heparin. We suggest that prothrombin ligands such as bothrojaracin may serve as prototypes for development of new zymogen-directed drugs that could result in prolonged half-life and possible decreased hemorrhagic risk.

2. Material and methods

2.1. Animals

Male and female Wistar rats and Balb/c mice were housed under controlled conditions of temperature (24 ± 1 °C) and light (12 h light starting at 7:00 a.m.). All animal care and experimental protocols were conducted following the guidelines of the institutional care and use committee (Committee for Evaluation of Animal Use for Research from the Federal University of Rio de Janeiro, CAUAP-UFRJ) and the NIH Guide for the Care and Use of Laboratory Animals (ISBN 0-309-05377-3). The protocols were approved by CAUAP-UFRJ under registry #IBQM/081-05/16. Technicians dedicated to the animal facility at the Instituto de Bioquímica Médica Leopoldo de Meis/UFRJ (RJ, Brazil) carried out all aspects related to rat and mouse husbandry under strict guidelines to insure careful and consistent handling of the animals.

2.2. Materials and drugs

Lyophilized *Bothrops jararaca* venom was kindly provided by Instituto Butantan (São Paulo, SP, Brazil). Bothrojaracin was purified as previously described (Arocas et al., 1996). Human α-thrombin was purified from frozen human plasma samples following a previously published method (Ngai and Chang, 1991). Rabbit serum containing polyclonal antibodies raised against purified bothrojaracin was developed as described (Castro et al., 1999). Anti-rabbit IgG alkaline phosphatase conjugates, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were from Sigma (St Louis, MO); Polyvinylidene fluoride (PVDF) membranes were from Millipore Corporation (Bedford, MA). All other reagents were of analytical grade. Reagents for determination of APTT (cephalin plus kaolin) and PT (thromboplastin with calcium) were from Bio-Mériaux (RJ, Brazil). Anasedan (Xylazin) and Dopalen (Ketamin) were from Agribrands (RJ, Brazil). Silicone tubing (0.9 × 25 mm) BD Insytet was purchased from Dickinson Ind. Cirúrgicas (MG, Brazil).

2.3. Polyacrylamide gel electrophoresis and western blot assays

Complex formation between bothrojaracin and prothrombin in rat or mouse plasma was detected as previously described (20). Bothrojaracin was incubated with rat or mouse plasma for 15 min and further submitted to 12.5% polyacrylamide gel. Electrophoresis was performed under non-denaturing conditions. For Western blot assays, proteins were electrotransferred onto PVDF membranes using a Semi-dry Electrotransferring System (Bio-Rad, USA), according to the instructions of the manufacturer. Membranes were then developed using anti-bothrojaracin polyclonal antibodies followed by incubation with the appropriate anti-IgG alkaline phosphatase conjugate. Staining was obtained by using the NBT/BCIP system (Sigma) according to the instructions of the manufacturer.

2.4. Stasis-induced thrombosis

Venous thrombosis was performed in a rat model as described (Vogel et al., 1989) with slight modifications (Mendes-Silva et al., 2003). Thrombus formation was induced by a combination of stasis and hypercoagulability. Animals were previously anesthetized as described above and the carotid artery was carefully exposed and dissected free from surrounding tissue. Bothrojaracin or phosphate-buffer (PBS) pH 7.4 (50 μL, final volume) was injected through a silicone tubing inserted into the carotid artery before the thrombosis induction. Then, the abdomen was surgically opened, and after careful dissection the vena cava was exposed and dissected free from surrounding tissue. Two loose ligatures were prepared 1 cm apart on the inferior vena cava just below the left renal vein. The thrombosis was induced by thromboplastin (3 mg/kg, diluted in PBS/50 μL) injected into the vena cava and stasis was immediately established by tightening the proximal suture. Tightening of the distal suture was performed 20 min after administration of thromboplastin and the ligated segment was removed. The formed thrombus was removed from the segment, rinsed, blotted on filter paper, dried for about 1 h at 60 °C, and weighed.

2.5. Bleeding effect

Evaluation of the bleeding effect was assessed by a tail transaction method (Herbert et al., 1996). Rats were anesthetized as described above and the carotid artery was carefully exposed and dissected free from surrounding tissue. A cannula was inserted into the right carotid artery and the same procedure was accomplished for components administration (50 μL, final volume). After 5 min of compounds administration the rat's tail was cut 3 mm from the tip. The tail was carefully immersed in 40 mL of distilled water at room temperature. Blood loss was evaluated 60 min later as a function of absorbance at 540 nm due to the hemoglobin content in water solution. The absorbance detected for a group that received PBS was taken as negative control for blood loss. In some cases, animals received heparin (1 mg/kg) as positive control for blood loss.

2.6. Thrombin-induced pulmonary thromboembolism

Antithrombotic effect of bothrojaracin was evaluated in a thrombin-induced pulmonary thromboembolism model in mice. Thrombotic event was induced by thrombin (2000 UI/kg), and bothrojaracin was injected i.v. at retro-orbital venous plexus 30 or 60 min before the thrombogenic stimulus. PBS (pH 7.4) was used as negative control. Animals still alive after 15 min were considered to be survivors.